Vol. 193, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1993 Pages 133-138

PLASMA GLUTATHIONE PEROXIDASE REDUCES PHOSPHATIDYLCHOLINE HYDROPEROXIDE

Yorihiro YAMAMOTO, Yuichiro NAGATA, Etsuo NIKI*,
Keiichi WATANABE**, and Shinichi YOSHIMURA***

Department of Reaction Chemistry, Faculty of Engineering,
University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113

*Research Center for Advanced Science and Technology,
University of Tokyo, Komaba, Meguro-ku, Tokyo 153

**Department of Pathology and ***Department of Molecular Life Science (Cell Biology), School of Medicine, Tokai University, Isehara, Kanagawa 259-11, Japan

Received April 12, 1993

Summary: The reducing activity of rat plasma glutathione peroxidase on phosphatidylcholine hydroperoxide (PC-OOH) and cholesteryl ester hydroperoxide (CE-OOH) was examined since these hydroperoxides are the major oxidation products of plasma. PC-OOH was reduced by the enzyme while CE-OOH was not. The reduction of PC-OOH by the enzyme ceased when all thiol was consumed, but the activity was recovered by the addition of glutathione, suggesting glutathione is important to keep the enzyme in the reduced form. These results are consistent with the findings that CE-OOH is present in human and rat plasmas while PC-OOH is undetectable and suggest that one of the physiological roles of the enzyme is to reduce PC-OOH.

Oxidative stress has been suggested to be connected with aging and degenerative diseases such as rheumatoid arthritis, reperfusion injury, cardiovascular disease, immune injury, and cancer [1]. The presence of cholesteryl ester hydroperoxide (CE-OOH) in human and rat plasmas [2,3] is one evidence of oxidative stress *in vivo*. When plasma or low density lipoprotein are exposed to oxygen radicals, CE-OOH and phosphatidylcholine hydroperoxide (PC-OOH) are the major oxidation products [4-6]. However, there is no detectable PC-OOH in human and rat plasmas [2,3,7,8]. In order to understand the reason for this

0006-291X/93 $4.00

difference, we have studied the reduction of these hydroperoxides by rat plasma glutathione peroxidase.

Glutathione peroxidase (GSH Px) is regarded as one of the major defenses against oxidative stress [1,9], since it can reduce hydrogen peroxide and organic hydroperoxides that otherwise can initiate oxidation of lipids, proteins, sugars, and other biological components following their interaction with transition metal ions [1]. Three types of GSH Px have been reported and all of them are selenium enzymes. The "classical" GSH Px found in red blood cell, liver, and other intracellular spaces [9,10] can reduce hydrogen peroxide and free fatty acid hydroperoxides but not PC-OOH [9,11,12]. Ursini *et al.* isolated the second intracellular GSH Px and found that it can reduce PC-OOH [11]. It was named phospholipid hydroperoxide glutathione peroxidase [12]. Thirdly, from the extracellular space, a plasma GSH Px was isolated [13-15] and characterized [13-19]. This plasma GSH Px can also reduce hydrogen peroxide and free fatty acid hydroperoxides [15].

The reduction of hydroperoxides by these three enzymes was studied in the presence of milimolar concentration of glutathione (GSH). This is reasonable for intracellular enzymes because milimolar GSH is present in these spaces. However, plasma levels of GSH are very low (about 0.3 μM [20]). Therefore, the physiological role of plasma GSH Px is not clear and the effect of GSH on the activity of plasma GSH Px should be studied more carefully. In this paper, we would like to present experimental evidence that plasma GSH Px can reduce PC-OOH but not CE-OOH. The physiological role of the enzyme *in vivo* is also discussed.

MATERIALS AND METHODS

Soybean phosphatidylcholine, cholesteryl linoleate, linoleic acid, NADPH, GSH, cumene hydroperoxide, and glutathione reductase were purchased from Sigma. Phenyl Sepharose and Sephacryl S-200 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Solvents and other reagents were of the highest grade commercially available.

Plasma GSH Px was separated from rat serum using a phenyl Sepharose column and a Sephacryl S-200 column as described previously [17]. Fractions which decompose cumene hydroperoxide were concentrated by an Amicon Ultrafiltration Membrane Corn CF25 and stored at -80°C. 2-Mercaptoethanol was present during all the purification steps to preserve enzyme activity. Enzyme activity was measured by the oxidation of NADPH in the presence of 0.23 mM cumene hydroperoxide, 0.25 mM GSH, and 0.12 mM NADPH at 37°C in air. One unit of the enzyme activity is defined as 1 μmol NADPH oxidized/min at 37°C.

PC-OOH, cholesteryl linoleate hydroperoxide, and linoleic acid hydroperoxide (18:2-OOH) were prepared by the autoxidation of soybean phosphatidylcholine, cholesteryl linoleate, and linoleic acid, respectively, in the presence of α-tocopherol.

Hydroperoxides were purified by a semipreparative column as described previously [21].

Hydroperoxides were incubated with rat plasma GSH Px in phosphate buffered saline (pH 7.5) containing 100 μM EDTA at 37°C in air. The concentration of hydroperoxides was analyzed by a chemiluminescence-based HPLC-assay [2,21] by injecting reaction solution directly onto the HPLC column. HPLC columns and mobile phases for the analysis of PC-OOH, CE-OOH, and 18:2-OOH were respectively a silica gel column and methanol/40 mM monobasic sodium phosphate (9/1, v/v), an octylsilyl column and methanol/*tert*-butyl alcohol (19/1, v/v), and an aminopropyl column and methanol/40 mM monobasic sodium phosphate (9/1, v/v). The concentration of 2-mercaptoethanol was measured by an HPLC system equipped with an electrochemical detector.

RESULTS AND DISCUSSION

Figure 1 shows the decay of about 10 μM each of 18:2-OOH and PC-OOH by 2.6 units/ml of rat plasma GSH Px in the presence of 1 mM GSH. Similar to the earlier findings [15], 18:2-OOH was reduced by this enzyme and disappeared in a minute. The formation of hydroxy linoleic acid (18:2-OH) was confirmed by HPLC analysis after the conversion to a fluorescent derivative [22]. PC-OOH was also reduced by the enzyme to its alcohol, PC-OH (Fig. 1). The reduction of PC-OOH or 18:2-OOH was not observed in the absence of the enzyme. The formation of PC-OH was confirmed by its retention time using HPLC analysis and its absorbance at 234 nm. On the other hand, cholesteryl linoleate hydroperoxide was not reduced by plasma GSH Px (data not shown).

It is interesting that PC-OOH was reduced by plasma GSH Px even in the absence of GSH (Fig. 1). But, this may be ascribed to the presence of about 150 μM 2-mercaptoethanol used as the preservative of the enzyme. The specificity of reductants for the enzyme is currently under investigation.

Since the peptide loops constituting the active site are strongly conserved among "classical" GSH Px, phospholipid hydroperoxide GSH Px, and plasma GSH Px [18], plasma GSH Px is likely to show a similar kinetic behavior with other GSH Px. The "pin pong" mechanism consisting of three steps has been proposed for the catalytic reaction of "classical" and phospholipid hydroperoxide GSH Px [12,23-25]. The first step of the catalytic cycle is the oxidation of selenol anion (E-CysSe$^-$) by PC-OOH to yield a selenenic acid derivative (E-CysSeOH) and PC-OH (eq. 1). A selenenic acid derivative is reduced by two molecules of GSH with intermediary formation of a selenosulfide (E-CysSe-SG) between the enzyme and GSH (eqs. 2 and 3).

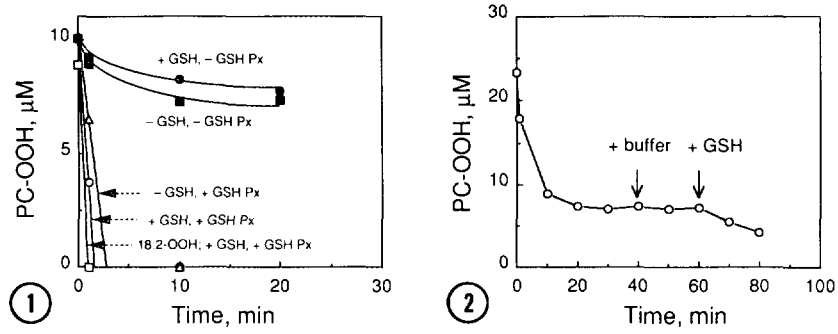

Fig. 1. Reduction of phosphatidylcholine hydroperoxide (PC-OOH) and linoleic acid hydroperoxide (18:2-OOH) by 2.6 units/ml rat plasma glutathione peroxidase containing 150 μM 2-mercaptoethanol as a preservative in phosphate buffered saline containing 100 μM EDTA in the presence of 1 mM glutathione (GSH) at 37°C in air. The stability of PC-OOH in the absence of the enzyme, GSH, or both is also shown.

Fig. 2. Reduction of 23 μM PC-OOH by 0.22 units/ml of rat plasma glutathione peroxidase in phosphate buffered saline containing 100 μM EDTA in the absence of GSH but in the presence of about 14 μM 2-mercaptoethanol at 37°C in air. Phosphate buffered saline and 0.6 mM GSH as a final concentration were added at the time indicated by the arrows.

$$\text{E-CysSe}^- + \text{H}^+ + \text{PC-OOH} \longrightarrow \text{E-CysSeOH} + \text{PC-OH} \quad (1)$$

$$\text{E-CysSeOH} + \text{GSH} \longrightarrow \text{E-CysSe-SG} + \text{H}_2\text{O} \quad (2)$$

$$\text{E-CysSe-SG} + \text{GSH} \longrightarrow \text{E-CysSe}^- + \text{GSSG} + \text{H}^+ \quad (3)$$

Figure 2 demonstrates the reduction of PC-OOH by 0.22 units/ml rat plasma GSH Px in the absence of GSH but in the presence of 14 μM 2-mercaptoethanol. The PC-OOH-reducing activity ceased when all 2-mercaptoethanol was consumed but the activity was recovered by the addition of GSH, indicating the oxidized form of the enzyme can be reduced by GSH. These results also support the "pin pong" mechanism. It is noteworthy that the addition of buffer had no effect.

The level of plasma GSH Px in humans is about 0.2 units/ml [Yoshimura S., *et al.*, unpublished data]. If we take a specific activity of the enzyme as 125 units/mg [9] and the molecular weight of the subunit as 23000 [10], the concentration of selenium is; 0.2/125/23000 = about 70 nM. In other words, plasma has a capacity to reduce 70 nM hydroperoxide without consuming GSH and the presence of 300 nM GSH will enlarge the hydroperoxide-reducing capacity. It is important that the concentration of GSH is much higher than the level of hydroperoxide detected in human plasma, i. e.,3 nM CE-OOH.

In summary, it was shown that rat plasma GSH Px can reduce PC-OOH but not CE-OOH. This is consistent with the presence of CE-OOH and the absence of PC-

OOH in human and rat plasmas, and suggests the important role of plasma GSH Px in reducing PC-OOH *in vivo*.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Cancer Research from the Ministries of Education, Science and Culture, and Health and Welfare of Japan, and by Watanabe Memorial Foundation.

REFERENCES

1. For example, Halliwell, B. and Gutteridge, J. M. C. (1989) Free Radicals in Biology and Medicine, 2nd Ed., Clarendon Press, Oxford.
2. Yamamoto, Y. and Niki, E. (1989) Biochem. Biophys. Res. Commun., **165**, 988-993.
3. Yamamoto, Y., Wakabayashi, K., Niki, E., and Nagao, M. (1992) Biochem. Biophys. Res. Commun., **189**, 518-523.
4. Frei, B., Stocker, R., and Ames, B. N. (1988) Proc. Natl. Acad. Sci. USA, **85**, 9748-9752.
5. Stocker, R., Bowry, V. W., and Frei, B. (1991) Proc. Natl. Acad. Sci. USA, **88**, 1646-1650.
6. Yamamoto, Y., Kawamura, M., Tatsuno, K., Yamashita, S., Niki, E., and Naito, C. (1991) Oxidative Damage and Repair. Ed. by Davies, K. J. A., Pergamon Press, Oxford, pp 287-291.
7. Yamamoto, Y. and Ames, B. N. (1987) Free Radical Biol. Med., **3**, 359-361.
8. Frei, B., Yamamoto, Y., Niclas, D., and Ames, B. N. (1988) Anal. Biochem., **175**, 120-130.
9. Flohe, L. (1982) Free Radicals in Biology. Ed. by Pryor, W. A., Academic Press, New York, pp 223-254.
10. Tappel, A. L., Hawkes, W. C., Wilhelmsen, E. C., and Motsenbocker, A. (1984) Methods Enzymol., **107**, 602-619.
11. Ursini, F., Maiorino, M., Valente, M., Ferri, L., and Gregolin, C. (1982) Biochim. Biophys. Acta, **710**, 197-211.
12. Ursini, F., Maiorino, and Gregolin, C. (1985) Biochim. Biophys. Acta, **839**, 62-70.
13. Takahashi, K., Avissar, N., Within, J., Cohen, H. (1987) Arch. Biochem. Biophys., **256**, 677-686.
14. Broderick, D. J., Deagen, J. T., and Whanger, P. D. (1987) J. Inorg. Biochem., **30**, 299-308.
15. Maddipati, K. R. and Marnett, L. J. (1987) J. Biol. Chem., **262**, 17398-17403.
16. Takahashi, K., Akasaka, M., Yamamoto, Y., Kobayashi, C., Mizoguchi, J., and Koyama, J. (1990) J. Biochem., **108**, 145-148.
17. Yoshimura, S., Watanabe, K., Suemizu, H., Onozawa, T., Moriguchi, J., Tsuda, K., Hatta, H., and Moriuchi, T. (1991) J. Biochem., **109**, 918-923.
18. Schuckelt, R., Brigelius-Flohe, R., Maiorino, M., Roveri, A., Reumkens, J., Straßburger, W., Ursini, F., Wolf, B., and Flohe, L. (1991) Free Radical Res. Commun., **14**, 343-361.
19. Chu, F.-F., Esworthy, R. S., Doroshow, J. H., Doan, K., and Liu, X.-F. (1992) Blood, **79**, 3233-3238.

20. Wendel, A. and Cikryt, P. (1980) FEBS Lett., **120**, 209-211.
21. Yamamoto, Y., Brodsky, M. H., Baker, J. C., and Ames, B. N. (1987) Anal. Biochem. **160**, 7-13.
22. Nagata, Y., Yamamoto, Y., Yamashita, S., and Niki, E. (1992) Oxygen Radicals, Eds. by Yagi, K., Kondo, M., Niki, E., and Yoshikawa, T., Elsevier, Amsterdam, pp 315-318.
23. Flohe, L. and Loschen, G., Gunzler, W. A., and Eichele, E. (1972) Hopp-Seyler's Z. Physiol. Chem., **353**, 987-999.
24. Chaudiere, J. and Tappel, A. L. (1983) Arch. Biochem. Biophys., **226**, 448-457.
25. Epp, O., Ladenstein, R., and Wendel, A. (1983) Eur. J. Biochem., **133**, 51-69.